PATENT
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Applicant(s) :	Wu et al.	)	Examiner: C. Collins
Serial No. :	RCE of 09/350,393	)	
Cnfrm. No. :	7999	)	Art Unit: 1638
Filed :	July 9, 1999	)	
For :	METHOD OF MAKING WATER STRESS OR SALT STRESS TOLERANT TRANSGENIC CEREAL PLANTS	)	

DECLARATION OF RAY J. WU UNDER 37 C.F.R. § 1.132

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I, RAY J. WU, hereby declare:

1. I received a Bachelor of Science degree in Chemistry from the University of Alabama in 1950 and a Ph.D. in Biochemistry from the University of Pennsylvania in 1955.

2. I am currently Liberty Hyde Bailey Professor of Molecular Biology and Genetics at Cornell University, International Professor of Molecular Biology and Genetics at Cornell University, and Professor of Biochemistry at Cornell University.

3. I am a member of the American Society of Biological Chemists, the American Association for the Advancement of Science, the American Chemical Society, Alpha Chi Sigma, the International Society for Plant Molecular Biology, the Tissue Culture Association, and the Rice Genetics Cooperative. I am a fellow of the American Association for the Advancement of Science and am a member of the editorial board of Method.

4. I am a joint inventor of the above-identified application.

5. I am submitting this declaration to: (1) describe the disclosure of Wu et al., "Production of Transgenic Rice Plants that are Resistant to Insect Pests and Fungal Diseases

or to Water and Salt Stress," General Meeting of the International Program on Rice Technology, abstract 113 (1997) ("Wu I"); and (2) describe the unexpected results achieved with the composite promoter of my present invention.

Disclosure of Wu I

6. Wu I is an abstract that discloses using constitutive or ABA-inducible promoters to drive water stress or salt stress tolerance genes in transgenic rice plants. Wu I does not teach or suggest an abscisic acid response complex ("ABRC") composite promoter, which includes at least one ABRC unit and a minimal promoter necessary and sufficient for promoter activity linked together to permit expression of a DNA molecule in the leaves or roots of a plant, as claimed in my present application. In particular, the disclosure of an ABA-inducible promoter in Wu I would not necessarily teach or suggest a composite promoter, as claimed in my present application. For example, numerous non-composite, ABA-inducible promoters exist in nature. However, a naturally occurring ABA-inducible promoter does not necessarily function in the same way as an ABRC composite promoter, as claimed in my present application. In particular, a naturally occurring ABA-inducible promoter, such as the Hva22 promoter from barley, is a seed specific ABA-inducible promoter. Thus, this naturally occurring ABA-inducible promoter is not suitable for driving the expression of a foreign gene to confer stress tolerance to a plant. This is because, in order to make a plant tolerant to abiotic stresses, the DNA molecule that increases tolerance to salt stress and drought stress needs to be expressed in the leaves or roots of the plant.

7. In contrast, a composite promoter including an ABRC unit and a minimal promoter necessary and sufficient for promoter activity, as claimed in my present application, functions in the leaves and/or roots of a transgenic plant. Thus, the inserted DNA molecule that increases tolerance to salt stress and drought stress in plants can be expressed in the leaves and/or roots of the transgenic plant to protect the plant from these abiotic stresses in my claimed invention.

The Unexpected Results Achieved by the Use of a Composite Promoter in Accordance with my Present Invention

8. It is my understanding that the claims of my above-identified application are rejected for obviousness over Xu et al., "Expression of a Late Embryogenesis Abundant Protein Gene, *HVA1*, from Barley Confers Tolerance to Water Deficit and Salt Stress in Transgenic Rice," Plant Physiol., 110:249-257 (1996) ("Xu") in view of Shen et al., "Modular Nature of Abscisic Acid (ABA) Response Complexes: Composite Promoter Units That Are Necessary and Sufficient for ABA Induction of Gene Expression in Barley," The Plant Cell, 8:1107-1119 (1996) ("Shen"), and further in view of the admitted prior art that *Agrobacterium*-mediated transformation of monocotyledonous plants was known in the art at the time of my present invention.

9. Xu discloses introducing the *HVA1* gene from barley into rice cells using the biolistic-mediated transformation method to generate transgenic rice plants. Xu teaches that expression of the *HVA1* gene regulated by the rice actin 1 gene promoter led to high-level, constitutive accumulation of the HVA1 protein in the transgenic rice plants which exhibited significantly increased tolerance to water deficit and salt stress.

10. Shen discloses the sequence for an ABRC from a barley *HVA1* gene. Shen teaches that the combination of different ACGT-boxes and coupling elements leads to the formation of ABRCs with different transcription strengths in a transient assay. This reference also suggests that the disclosed synthetic promoters (including an ABRC and the Amy64 minimal promoter) capable of conferring different levels of ABA induction could be used to drive the expression of genes that would enhance plant stress tolerance.

11. However, it can not be determined from the disclosures of Xu and Shen whether the use of a minimal promoter linked to an ABRC unit, as claimed in my present application, would be able to confer tolerance to water or salt stress in transgenic plants, let alone achieve results at least equivalent to those of the constitutive promoter of Xu, which is identified in Xu as producing transgenic plants showing "significantly increased tolerance to water deficit and salinity" (see Abstract). In particular, a showing of ABA-induction alone for a particular composite promoter in a transient assay, as in Shen, does not teach or suggest conferring salt or water stress tolerance in transgenic plants, since not all ABA-inducible promoters are suitable for driving the expression of a foreign gene to confer stress tolerance to a plant (see, e.g., ¶ 6, *supra*).

Moreover, it was necessary to experimentally determine whether a minimal promoter linked to an ABRC unit, as claimed in my present application, would confer suitable tolerance to water or salt stress in transgenic plants.

12. As set forth in my present application and below, it has unexpectedly been determined that transgenic plants transformed with a DNA molecule that increases tolerance to salt stress and drought stress in plants driven with a composite promoter including at least one ABRC unit and a minimal promoter exhibit improved tolerance to salt stress and drought stress as compared to transgenic plants transformed with a DNA molecule that increases tolerance to salt stress and drought stress in plants driven with a constitutive promoter.

13. In particular, multiple parallel experiments were performed using a constitutive promoter and a composite promoter of the present invention including at least one ABRC unit and a minimal promoter. One set of experiments is described in Examples 11 and 12 of my above-identified application (see Specification at page 26, line 39 to page 28, line 31 and page 18, line 5 to page 26, line 37 for a detailed description of production of plasmids, transgenic plants, and exposure to water or salt stress conditions). In particular, transgenic plants including a constitutive promoter (rice line JS102) and transgenic plants including a composite promoter of the present invention (which includes four ABRC units and a minimal promoter necessary and sufficient for promoter activity -- Act1-100) (rice lines JS112 and JS110) were subjected to water stress and salt stress conditions. As shown in Table 3 (see Specification at page 27, lines 19-41), under water stress (top half of Table 3) or salt stress (bottom half of Table 3) conditions, transgenic rice plants that used a composite promoter of the present invention to drive the expression of the Δ^1-pyrroline-5-carboxylate synthetase gene (rice line JS112) grew much faster as compared to plants that used a constitutive promoter (rice line JS102).

14. Moreover, a second set of experiments was conducted, in which it was unexpectedly determined that transgenic plants transformed with a DNA molecule that increases tolerance to salt stress and drought stress in plants driven with a composite promoter including at least one ABRC unit and a minimal promoter exhibit improved tolerance to salt stress and drought stress as compared to transgenic plants transformed with a DNA molecule that increases tolerance to salt stress and drought stress in plants driven with a constitutive promoter. In particular, when plants are being stressed by high salinity or drought conditions, the cell membranes become leaky and ionic compounds leak out of the cells. Extensive leakage of ions

and other cellular contents will lead to the death of cells and tissues. Therefore, the extent of leakage of ions is a measure of the health of the plant. The higher extent of leakage, the less healthy the plant. A common method for measuring the extent of leakage of ions is to determine the electrical conductivity of the water used to soak the leaves. This method was applied as described below.

15. Transgenic plants including a constitutive promoter (rice *Act1* promoter) and the *Hva1* gene (pRKJ6) were prepared, as described in Rohila et al., "Genetic Improvements of Basmati Rice for Salt and Drought Tolerance by Regulated Expression of a Barley *Hva1* cDNA," Plant Sci. 163:525-532 (2002) ("Rohila") (copy attached as Exhibit A). In addition, transgenic plants including a composite promoter of the present invention (which includes four ABRC units and a minimal promoter necessary and sufficient for promoter activity -- Act1-100) and the *Hva1* gene (pRKJ21) were prepared, as described in Rohila. Briefly, three-week old calli of rice cv. Pusa Basmati 1 were co-cultivated with *Agrobacterium* strain LBA4404 harboring pRKJ6 or pRKJ21. Selection of transformed calli and regeneration of plantlets were carried out as described in Rohila. The transgenic plants were then subjected to salt stress conditions and electrical conductivity of the resulting plants was measured. In particular, mature seeds harboring a single copy of the transgene were selected from independent transgenic R2 rice lines from each plasmid (i.e., RKJ6 and RKJ21). These seeds, as well as seeds from non-transgenic and untransformed controls, were sown on Petri dishes lined with moist filter paper. Later on, the plants were grown for three weeks in soil in the greenhouse. For determination of salt stress, three-week-old plants were irrigated daily with 200 mM saline water to maintain the salinity level. After eight days of stress, salt was flushed out by heavy irrigation of pots with tap water twice a day for two days. Then, two additional cycles of salt stress were carried out. After completion of the salt stress, the next leaf to the flag leaf was removed and cut into small pieces. The pieces of leaves were immediately put into a test tube containing 2.5 ml cold water, and gently vortexed for 15 seconds. The tubes were placed in a dessicator, and a vacuum was applied for five minutes to remove air bubbles from the surface of leaf tissues. The tubes were then covered and placed at room temperature for 24 hours. A VWR conductivity meter with a platinum electrode and built-in temperature correction was used to measure the electrical conductivity of the solution. The results are shown in Table 1, below.

Table 1. Electrical conductivity readings from R2 plant leaves after salt stress

Plasmid	Line #	Electrical conductivity (μmho/mg leaf)			
		Average value[a] per plant line	Average of 4 transgenic lines	%[b]	Average % of 4 transgenic lines
pRKJ6 (*Act1-Hva1*)	42	3600 ± 60		63	
	44	3830 ± 186	4020	67	70.0
	220	4540 ± 138		79	
	289	4110 ± 107		71	
	Neg[c]	5755 ± 65		100	
	NT[d]	5850 ± 35		102	
pRKJ21 (*4 ABRC-Hva2*)	12	3160 ± 80		55	
	13	3360 ± 78	3435	58	59.5
	14	3570 ± 40		62	
	96	3650 ± 120		63	
	Neg[c]	5795 ± 145		100	
	NT[d]	5920 ± 300		102	

[a]The values are mean from three replicates in each line.
[b]Indicates the percentage of the electrical conductivity of each transgenic plant line and NT over that of Neg control plants, which is set as 100%.
[c]Neg is non-expressing, transgenic line as a control.
[d]NT is untransformed control.

16. As shown in Table 1, *Hva1*-producing transgenic plants driven by the composite promoter of my present invention were healthier, as reflected by lower extents of ion leakage from leaves under salt stress, compared to those plants with *Hva1* driven by a constitutive promoter (Act1 promoter).

17. In a third set of experiments, production of glycine betaine in transgenic rice plants harboring the choline oxidase gene (*Cox*) from *Arthrobacter pascens* was tested. Transgenic plants where *Cox* was driven by a composite ABRC-inducible promoter of the present invention (including four ABRC units and a minimal promoter necessary and sufficient for promoter activity -- Act1-100 (identical to pJS112, except the P5CS cDNA is replaced by the Cox cDNA)) and transgenic plants where *Cox* was driven by a constitutive promoter (ubiquitin promoter) were produced using the same general methodology as described the Examples of my above-identified patent application. 24-day old rice plants were stressed with 150 mM NaCl for six days, watered for seven days, and then stressed with 150 mM NaCl for 12 days. Plant

biomass was measured after watering for ten days. The results are shown in Table 2, below.

Table 2
Salt stress tolerance test of *Cox* transgenic rice plants.*

Plants	Promoter Used to Drive *Cox*	Fresh Root Weight§	Fresh Shoot Weight§
Non-transgenic	None	0.21	2.2
		0.24	2.4
Transgenic	Inducible (ABRC)	0.9	6.0
		1.2	6.8
		1.3	7.7
Transgenic	Constitutive (ubiquitin)	0.4	3.2
		0.5	3.7
		0.6	3.7

**Cox* is the choline oxidase gene from *Arthrobacter pascens*.
§Gram per plant. Average of 6 plants per line.

18. As shown in Table 2, transgenic rice plants grew at least twice as fast after salt stress when the *Cox* gene was driven by an ABRC-inducible composite promoter of my present invention, as compared to plants where the *Cox* gene was driven by a constitutive promoter.

19. Accordingly, in all three sets of parallel experiments using a constitutive promoter and a composite promoter of my present invention, the composite promoter of my present invention unexpectedly resulted in improved tolerance of transgenic plants to salt stress and drought stress.

20. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: February 26, 2004

Ray J. Wu

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Genetic improvement of Basmati rice for salt and drought tolerance by regulated expression of a barley *Hva1* cDNA

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Abstract

An *HVA1* gene from barley was introduced and expressed under the control of a constitutive or a stress-inducible promoter in a recalcitrant scented rice variety, Pusa Basmati 1 to increase the tolerance against abiotic stresses. Third generation (R2) transgenic plants, each harboring a single copy of intact transgene expression cassette, were tested for stress tolerance. Homozygous transformants were exposed to high salinity or drought stress. These transgenic lines showed increased stress tolerance in terms of cell integrity and growth after the imposed salt- and water-stress treatments, compared to the control plants. The results showed that high levels of LEA3 accumulation in the leaves of transgenic Pusa Basmati 1 rice plants might have conferred the significant increase in tolerance against drought and salt stress.

Keywords: *Actin1* promoter; *Hva1*; Stress-inducible promoter; Stress tolerance; Transgenic Basmati rice

1. Introduction

Aromatic rice varieties are economically important as they receive a premium price in agricultural markets worldwide [1]. The most prominent examples are Jasmine and Basmati rice. Basmati rice is well known for its exquisite aroma and grain quality [2]. Environmental stresses due to high concentrations of salt in soils and limited water availability are among the most serious factors limiting productivity in Basmati rice. Moreover, abiotic constraints (salinity, drought) are more prominent and yield limiting in rice than biotic constraints [3–6]. Even with good water management, seasonal increases in topsoil salinity and periodic drought spells can hardly be avoided. Basmati rice cultivation has been restricted to irrigated soils only and the marginal lands in the Basmati rice belt are either less productive or totally un-utilized for Basmati rice cultivation. Basmati rice breeding has been difficult due to the complex nature of quality traits and poor combining ability of Basmati rice varieties [1,7]. Crosses between Basmati rice (Group V) and improved indica (Group I) are incompatible causing inter-group hybrid sterility. Attempts to introduce agronomically useful genes into the Basmati rice background met with limited success. The lack of success in breeding stress-tolerant varieties would suggest that conventional breeding practices alone are not sufficient. Genetic transformation provides novel opportunities for the transfer of agronomically useful genes in an elite cultivar without disturbing its genetic background. Genes for several important traits are now available and can be transferred into Basmati rice to improve its resistance/tolerance against biotic and abiotic stresses [8,9]. Hence, the development of genetically engineered Basmati plants with improved agronomical characteristics presents a current challenge in crop biotechnology [8–11]. Our goal is to improve Pusa Basmati 1 rice against drought and salt stress by introducing an *HVA1* gene (*Hva1*) by genetic transformation. Earlier workers have demonstrated an improvement in abiotic stress tolerance of Japonica rice by overproducing an HVA1 protein

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(LEA3) [12], an LEA2 and LEA1 protein [13], proline [13] or polyamines [14].

LEA proteins are among the best known of water-stress-induced proteins. Most *Hva1* are responsive to abscisic acid (ABA). Xu et al. [12] transferred a barley *Hva1* driven by a constitutive promoter to a Japonica rice variety, Nipponbare, using the biolistic transformation procedure. The transgenic plants showed increased tolerance to both water and salt stresses. While constitutive promoters give certain advantages in various situations, the level of transgene expression is often low or variable, especially in R1 and R2 generation plants. This is most likely due to gene silencing, which is potentially related to high copy number of intact and rearranged transgenes. Another problem is that constitutive expression of certain transgenes under non-stress environments produces transgenic proteins that consume energy and building blocks, which may have negative impacts on normal growth of plants. Thus, it is desirable to produce plants that synthesize the required protein only under stress conditions. In this paper, we report the development and evaluation of a number of independent transgenic Basmati rice plant lines, each harboring a single copy of the *Hva1*, driven either by an ABA and stress-inducible promoter or by a constitutive promoter. We found that the transgenic Basmati rice plants showed increased tolerance against drought and high salinity.

2. Materials and methods

2.1. Construction of plasmids containing HVA1 gene

Two types of chimeric expression plasmids, pRKJ6, with barley *Hva1* driven by a constitutive rice *Act1* promoter [15], whereas pRKJ21, with *Hva1* driven by an abiotic stress-inducible promoter, were constructed. The stress-inducible promoter, *4ABRC–Act1*-100P-*Hva22* intron [16], is comprised of four copies of 49 bp ABA-responsive element (ABRE) from the barley *Hva22*, the rice actin minimal *Act1*-100 promoter (180 bp) and an *Hva22* intron (240 bp). The *Hva1* used in this study was a 1.0 kb fragment excised from pBY520 [10]. It was inserted in the polylinker region of the plasmids pRKJ17 and pRKJ19 to produce the two final constructs, pRKJ6 and pRKJ21, respectively, shown in Fig. 1. The vector was pCAMBIA 1200 that includes a 35S promoter and a hygromycin phosphotransferase gene (*hpt*) within the T-DNA (data not shown). Thus, hygromycin can be used for selection of transgenic plants.

Since the pCAMBIA vector lacks super-virulent genes and do not give high transformation frequencies in recalcitrant Basmati rice, we separately mobilized the two plasmids pRKJ6 and pRKJ21 into *Agrobacterium*

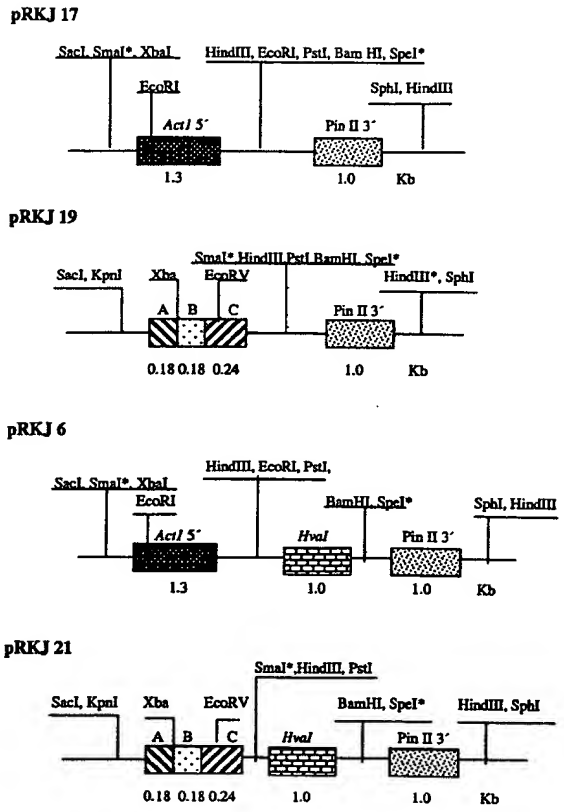

Fig. 1. Schematic diagrams and partial restriction maps of plasmids RKJ17, RKJ19, RKJ6 and RKJ21. The vector for all four plasmids was pCAMBIA 1200, which has a hygromycin resistance gene as a selectable marker. *Act1* 5′, rice *actin1* promoter with *actin1* first intron; pin II 3′, 3′ terminator region of potato proteinase inhibitor II gene; (A) 4 copies of ABRE; (B) *Act1* minimal promoter; (C) intron of barley *Hva22* gene; *Hva1*, late embryogenesis abundant protein (group 3) from barley. *Indicates unique restriction enzyme site.

tumefaciens strain LBA4404 carrying pSB1 by triparental mating. Plasmid SB1 lacks T-DNA but has all the virulence genes present in pTOK 233 [17] and contains a tetracycline resistance gene as selection marker.

2.2. Production of transgenic Basmati rice plants

For this study we used mature seed scutellum-derived embryogenic calli of rice cv. Pusa Basmati 1. Three-week-old calli were co-cultivated with *Agrobacterium* strain LBA4404 harboring pRKJ6 or pRJ21. Details of the entire procedure, including selection of transformed calli and regeneration of plantlets, were the same as described by Roy and Wu [14]. The only differences were that during selection maltose (30 g/l) was used in place of sorbitol and sucrose, and for selection hygromycin (50 mg/l) was used in place of bialaphos. In

addition, several minor improvements reported by Jain et al. [18] were included.

2.3. DNA-blot hybridization analysis of transgenic Basmati rice plants

Genomic DNA from transgenic rice plants was prepared as described by Zhao et al. [19]. Eight to ten micrograms of genomic DNA was digested with *SmaI* restriction enzyme (which cut only once in the plasmid DNA), electrophoresed through 1% agarose gels and transferred onto nylon membranes (Nytran Super Charge, Schleicher and Schuell Inc. Š. Keene, N.H.). Probe preparation, hybridization and detection were performed using the α^{32}P-dCTP radioactive labeling system following the manufacturer's (Gibco BRL) protocol.

2.4. Immunoblot analysis of LEA protein produced in transgenic Basmati rice plants

Leaves from 7- to 8-week-old, R2 transgenic plants after being subjected to salt and drought stresses, were used in immunoblot analysis to detect the presence of LEA3 protein. The procedure for immunoblot analysis was essentially as described by Xu et al. [12].

2.5. Evaluation of R2 transgenic lines for salinity and drought tolerance

Mature seeds harboring a single copy of the transgene were selected from independent transgenic R2 rice lines from each plasmid (i.e. RKJ6 and RKJ21). These seeds, as well as seeds from non-transgenic and untransformed controls, were sown on petri dishes lined with moist filter paper. Later on, the plants were grown for 3 weeks in soil in the greenhouse for determination of tolerance to either salt or dehydration stress. For determination of salt stress, 3-week-old plants were irrigated daily with 200 mM saline water to maintain the salinity level. After 8 days of stress, salt was flushed out by heavy irrigation of pots with tap water twice a day for 2 days. This represented the first stress cycle. Then, two additional cycles of salt stress were carried out. For determination of drought tolerance, 3-week-old, R2 transgenic, non-transgenic and untransformed controls were grown in pots where water was withheld for 7 days. The plants were then watered for 3 days for recovery. In this manner, two more cycles of stress were given before recording data on drought stress.

2.6. Plant growth performance of R2 plants after stress treatment

After three cycles of salt or drought stress, followed by three recovery periods, ten R2 plants from each transgenic line and control were measured for plant heights, fresh shoot weights and dry shoot weights. Here Guidelines from IRRI [20] were followed to measure different growth parameters. The dry shoot weights were measured after placing fresh shoots in a 120 °C oven for 4 h and then in an 80 °C oven for 48 h.

2.7. Ion leakage from leaf cells of R2 plants after salinity treatment

After completion of 200 mM NaCl salt stress, the next leaf to the flag leaf was removed and cut into small pieces. The pieces of leaves were immediately put into a test tube containing 2.5 ml cold water, and gently vortexed for 15 s. The tubes were placed in a desiccator, and a vacuum was applied for 5 min to remove air bubbles from the surface of leaf tissues. The tubes were then covered and placed at room temperature for 24 h [21]. Using leaves from non-stressed Pusa Basmati 1 plants, our preliminary results indicate that 24 h was an appropriate time to make measurements of relative conductivity of the solution in which the leaves were soaked. A VWR conductivity meter with a platinum electrode and built-in temperature correction was used to measure the electrical conductivity of the solution.

3. Results

3.1. Transformation efficiency

Transgenic Basmati rice plants that express the *Hva1* cDNA driven by a constitutive rice *actin1* promoter or a stress-inducible *ABRC* promoter were generated by *Agrobacterium tumefaciens*-mediated transformation. The components of the plasmids (pRKJ6 and pRKJ21) used in this study are shown in Fig. 1. Hundreds of hygromycin-resistant calli were obtained. Transformation efficiency was between 8 and 9%, which was calculated as percentage of total number of independent transgenic plant lines produced divided by the total number of calli used in co-cultivation. A total of 70 different independently transformed plants (R0) were produced (Table 1). The presence of the transgene was detected at several developmental stages in R1/R2 generations. In the segregating population (R1), the leaves of individual plants were cut and dipped into solidified MS medium containing hygromycin (100 mg/l). One week later, the resistant and sensitive phenotypes were scored to determine the Mendelian segregation pattern (data not shown). The leaves of resistant rice plants remained green, but leaves of sensitive plants turned yellow and eventually brown.

Table 1
A summary of data of Pusa Basmati 1 transformation experiments conducted using *Agrobacterium* LBA4404 strains containing *Hva1* cassette driven by either a rice *Actin1* (pRKJ6) or a *4ABRC* (pRKJ21) promoter

Plasmid	# of calli co-cultivated	# of calli survived	# of lines resistant to hygromycin	Transformation efficiency (%)	Fertile lines
pRKJ6 (*Act1-Hva1*)	500	257	40	8	40
pRKJ21 (*4ABRC-Hva1*)	350	150	30	9	30

3.2. DNA blot analysis of transgenic plants

Hygromycin-resistant plants were analyzed by Southern blot hybridization. DNA from various putative transgenic plants was digested with *SmaI* restriction enzyme and allowed to hybridize with the probe (α^{32}P-radiolabeled *Hva1*-pinII 3′ fragment). The resultant data on 12 plants are shown in Fig. 2. Since the T-DNAs of pRKJ6 and pRKJ21 contain a single *SmaI* site (Fig. 1), the number of hybridizing bands reflected the number of copies of the *Hva1* in the plant unless repeats of multiple copies of T-DNA had been integrated. Positive plants showed band(s) of more than 3.3 kb (in plants harboring *Act1–Hva1*) and 2.0 kb (in plants harboring *4ABRC–Hva1*), which are the minimum sizes of hybridizing fragments expected from the maps of pRKJ6 and pRKJ21, respectively. Plant lines showing an identical pattern of bands (such as plants 2 and 5 in Fig. 2) were considered clonal. Results from this figure showed that plants 1, 3, 4, 8, 9, 10, 11 and 12 each harbor a single copy of the transgene. However, plant 1 may be identical to plants 8 and 11, and plant 4 may be identical to plant 10.

3.3. Detection of HVA1 protein in leaf tissue

The accumulation of LEA3 in a number of individual transgenic R2 lines was detected by Western blot analysis. Fig. 3 shows the SDS-PAGE gel pattern. The presence of a 27 kDa band in most lanes, which corresponds to LEA3, indicates the presence of LEA3 in most of the R2 transgenic plants. After densitometer tracing of the bands, we found that the LEA3 levels after stress treatment were approximately twice as high in several of the R2 plants (such as plants 4 and 6 in Fig. 3a) where the *Hva1* was driven by the stress-inducible promoter, as opposed to those driven by the constitutive promoter (Fig. 3b). Protein extracts of non-transformed and negative plants did not show the 27 kDa LEA3 band. These data indicate that the *Hva1* present in

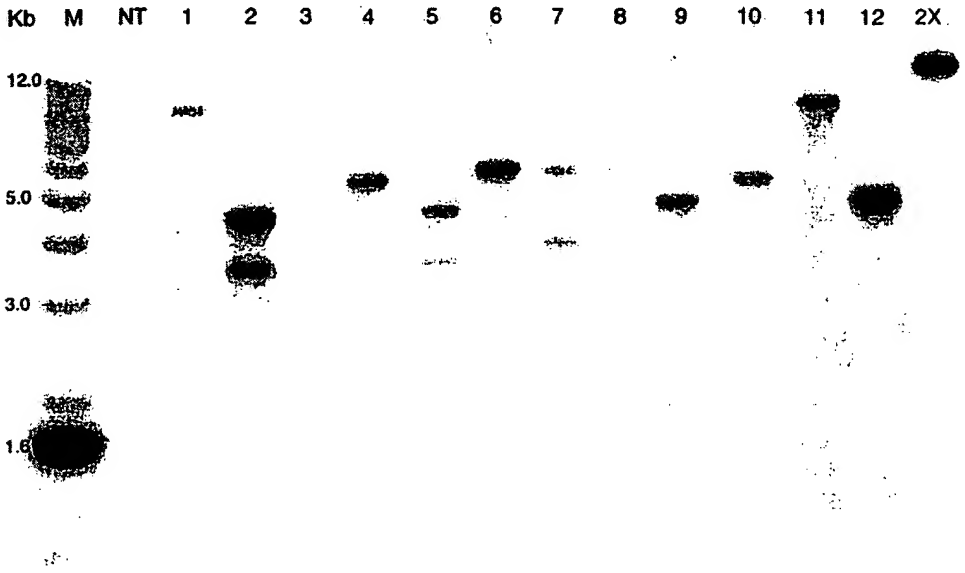

Fig. 2. Genomic DNA-blot analysis of different independent *Hva1* transgenic lines in R2 transgenic plants. Genomic DNA of each plant was digested with *SmaI* and fractionated by 1% agarose gel electrophoresis. A α-^{32}P-dCTP-labeled 2.0 kb HindIII fragment containing *Hva1* cDNA was used as the probe. M, DNA size marker (1 kb plus, Gibco BRL); NT, untransformed Basmati plant; 1–12, Different independent transgenic lines; 2X, *SmaI* digested plasmid DNA that was equivalent to approximately two copies of the transgene. Molecular weight standards are indicated in the left-hand side margin.

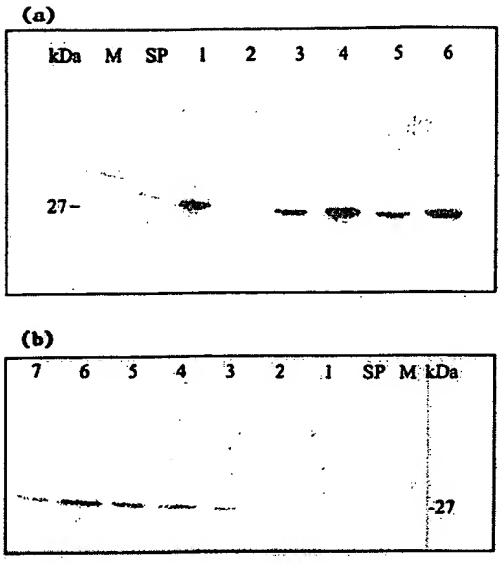

Fig. 3. Immunoblot detection of LEA3 accumulation in the leaves of R2 transgenic Basmati rice plants after salt stress. In each blot, equal amounts of total soluble proteins (20 μg) were separated on a 12% SDS-PAGE gel, and immunoblot detection was performed as described in materials and methods. A 27 kDa LEA3 protein band can be seen in transgenic *Hval*-expressing plants. M—molecular weight marker; SP—partially purified barley LEA3 overproduced in *E. coli*. (a) Samples from pRKJ21 plants. (b) Samples from pRKJ6 plants.

plasmids pRKJ6 and pRKJ21 encodes an active *Hval* and resulted in the production of the LEA3 in transgenic Basmati rice plants.

3.4. Stress analysis as measured by shoot height and shoot weight

Rice plants are very sensitive to salt, especially in the seedling stage [22]. Thus, in our study we used 3-week-old seedlings for drought- and salt-stress experiments. To test the effect of *Hval* expression on stress tolerance of seedlings, we used R2 seeds of four independent transgenic plants each for those in which the *Hval* was driven either by the rice *actin 1* or by the *4ABRC* promoter. Two types of control plants were used: untransformed Pusa Basmati 1-plants (NT), and the non-transgenic plants recovered from the transformation experiments (Neg). The seeds were germinated and then seedlings were subjected to the salt- or water-stress treatments. Results shown in Tables 2–4 indicate that expression of *Hval* in transgenic Basmati rice seedlings led to enhanced growth under either salt or drought stress as compared to plants that did not harbor an *Hval*. Enhanced growth was reflected by data on increase in shoot height (Table 2), fresh shoot weight (Table 3) and dry shoot weight (Table 4). For example, transgenic plants had 21–76% greater shoot height and 11–65% higher shoot weight compared to the non-transgenic control plants under either water-deficit or salt-stress conditions. Consistent results were obtained with seedlings from all four independent *Hval*-expressing lines. Transformed plants that did not harbor the barley *Hval* (Neg) showed growth characteristics that were almost identical to untransformed control plants (NT). The growth advantage of *Hval*-expressing seedlings during stress was transient, because after 2–3 weeks of recovery, the control seedlings and the *Hval*-expressing plants showed similar growth rates.

3.5. Intactness of cell membrane as reflected by ion leakage

Salt or drought stress results in damage of plant cell membrane and leakage of cellular contents. Leakage of ions is the easiest to measure by determining the electrical conductivity of the solution in which the leaves were soaked [21]. Results in Table 5 show that the electrical conductivity of samples from all transgenic plants is lower than those from non-transgenic plants. Transgenic plants that harbor an *Hval* driven by an inducible promoter (pRKJ21 plants) gave less ion leakage as compared to pRKJ6 plants, in which the *Hval* was driven by a constitutive promoter.

4. Discussion

Recent advances in plant biotechnology have provided biologists with the tools to engineer desirable traits into rice plants with capabilities far beyond those of conventional plant breeding techniques [23]. One important application of genetic transformation is to transfer one or more useful genes into an elite cultivar without disturbing its original genetic background. Any attempts made to introduce agronomically useful genes through cross hybridization into Basmati rice have met with limited success because of deterioration (or dilution) in the Basmati rice aroma and quality traits. These products have not been accepted by the consumer [1]. This makes Basmati rice an important candidate for genetic improvement by transformation. Basmati rice is quite sensitive to salinity and drought and is being cultivated in irrigated areas only. A number of stress-related genes have been isolated which can potentially improve the stress tolerance in plants [8]. Among them, LEA3 is well known for its accumulation during seed maturation and under environmental stress. Xu et al. [12] reported that barley *Hval* significantly improves the stress tolerance in a Japonica rice variety Nipponbare. In the present investigation, transgenic Basmati rice plants harboring *Hval* cDNA, driven by either a constitutive promoter or a stress-inducible promoter were produced and were analyzed for seedling/plant growth after being subjected to drought or salt stress.

Table 2
Average shoot height of R2 transgenic plants after dehydration or salt stress

Plasmid	Line #	Dehydration stress		Salt stress	
		Height[a] (cm)	%[b]	Height[a] (cm)	%[b]
pRKJ6 (*Act1-Hva1*)	42	30.8±0.99	158	29.2±0.74	153
	44	27.0±0.52	139	25.3±1.11	133
	200	23.9±0.64	123	24.1±0.96	127
	289	26.6±0.79	137	23.1±0.84	121
	Neg[c]	19.5±0.98	100	19.1±0.40	100
	NT[d]	19.7±0.60	101	19.3±0.60	101
pRKJ21 (*4ABRC-Hva1*)	12	31.4±0.98	160	31.0±0.76	176
	13	27.7±1.21	141	26.2±1.00	149
	14	24.3±1.10	124	23.4±0.91	133
	96	23.9±1.05	122	21.6±1.56	122
	Neg[c]	19.6±1.23	100	17.7±0.75	100
	NT[d]	19.7±0.60	100	19.3±0.60	109

[a] The values are mean from ten plants in each line.
[b] Indicates the percentage of the value of each transgenic plant line and NT over that of Neg control plants, which is set as 100%.
[c] Neg is a transformed but non-expressing, transgenic line used as a control.
[d] NT is an untransformed plant line used as a second control.

Labra et al. [24] reports heritable genomic changes in transgenic rice plants produced by *Agrobacterium*-mediated transformation. Thus, to rule out any possibility of stress tolerance through somaclonal variation, we used two types of control plants: untransformed Pusa Basmati 1 plants, and the plant lines obtained from the transformation experiments which did not contain the input *Hva1* cassette. In this study, the transgenic plants grew normally under non-stress conditions, and their growth was inhibited under salt- and water-deficit conditions although to lesser extent compared to the non-transgenic controls. It is clear that the response of plants to water and salt stress depends on the expression levels of responsible proteins and not by somaclonal variation or epigenetic effects. As shown in Table 2, the shoot height of *Hva1*-expressing, homozygous seedlings was substantially longer under both drought and salt-stress conditions, compared to those of control seedlings. Plants with *Hva1* driven by a stress-inducible promoter appeared to be healthier, as reflected by lower extent of ion leakage from leaves under salt stress conditions, compared to those plants with *Hva1* driven by a constitutive promoter (Table 5). These results show that transgenic plants with a stress-inducible promoter to drive the *Hva1* perform better than those driven by a constitutive promoter under salt-stress conditions. Our results are in agreement with those of Cheng et al. [13], who found a significant advantage in using an ABA-

Table 3
Fresh shoot weight of R2 transgenic plants after recovery from dehydration or salt stress

Plasmid	Line #	Dehydration stress		Salt stress	
		Average weight [a] (g)	%[b]	Average weight [a] (g)	%[b]
pRKJ6 (*Act1-Hva1*)	42	2.65±0.03	142	2.95±0.11	165
	44	2.61±0.12	139	2.56±0.10	143
	220	2.16±0.06	116	2.43±0.06	136
	289	2.37±0.07	127	2.51±0.09	141
	Neg[c]	1.87±0.04	100	1.79±0.06	100
	NT[d]	1.88±0.08	101	1.69±0.14	95
pRKJ21 (*4ABRC-Hva1*)	12	2.81±0.10	142	2.62±0.09	159
	13	2.77±0.12	140	2.52±0.06	153
	14	2.38±0.13	120	2.31±0.03	140
	96	2.21±0.13	111	2.29±0.06	138
	Neg[c]	1.98±0.11	100	1.65±0.10	100
	NT[d]	1.91±0.10	97	1.91±0.09	115

[a] The values are mean from five plants in each line.
[b] Indicates the percentage of the value of each transgenic plant line and NT over that of Neg control plants, which is set as 100%.
[c] Neg is non-expressing, transgenic line as a control.
[d] NT is untransformed control plant line.

Table 4
Dry shoot weight of R2 transgenic plants after recovery from dehydration or salt stress

Plasmid	Line #	Dehydration stress		Salt stress	
		Average weight [a] (g)	%[b]	Average weight [a] (g)	%[b]
pRKJ6 (*Act1-Hva1*)	42	0.66±0.14	204	0.65±0.03	164
	44	0.61±0.05	187	0.61±0.03	152
	220	0.54±0.03	165	0.52±0.03	131
	289	0.55±0.04	168	0.54±0.04	134
	Neg[c]	0.33±0.02	100	0.40±0.03	100
	NT[d]	0.37±0.02	113	0.37±0.03	92
pRKJ21 (*4ABRC-Hva1*)	12	0.71±0.02	177	0.60±0.03	154
	13	0.57±0.02	142	0.58±0.02	151
	14	0.56±0.03	140	0.57±0.03	147
	96	0.55±0.03	137	0.50±0.03	129
	Neg[c]	0.40±0.03	100	0.39±0.02	100
	NT[d]	0.39±0.06	199	0.38±0.04	99

[a] The values are mean from five plants in each line.
[b] Indicates the percentage of the value of each transgenic plant line and NT over that of Neg control plants, which is set as 100%.
[c] Neg is non-expressing, transgenic line as a control.
[d] NT is untransformed control.

inducible promoter compared to a constitutive promoter, for the expression of a gene *P5CS* that encodes an enzyme for synthesizing proline. The expression of this gene led to the accumulation of proline, and improved growth of transgenic rice plants under salt- and water-stress conditions. Similarly, after drought-stress treatments, stress-inducible *Hva1*-expressing plant lines showed less ion leakage as compared to those plants in which the *Act1* promoter was used to drive the expression of *Hva1*. These results further show that *Hva1* under the regulation of a stress-inducible promoter is capable of increasing stress tolerance of Basmati rice. Another advantage is that induction of the synthesis of the LEA3 only under stress conditions allowed the plants to grow normally under non-stress conditions.

Proteins in the LEA family are hydrophilic in nature, and thus, in vegetative tissues of transgenic plants, they bind more water than the control plants. This feature may result in preserving the intactness of cell membranes as reflected by a reduced amount of ion leakage from plants under stress. The data provide further evidence to support the role of LEA3 in dehydration-stress tolerance as shown in transgenic wheat [25].

In conclusion, we are able to transform a recalcitrant Basmati rice cultivar using the *Agrobacterium* system to produce fertile transgenic plants harboring a single or low-copy-number of functionally-intact transgene. Several of these transgenic Basmati rice plants showed increased tolerance towards salt and dehydration stress.

Table 5
Electrical conductivity readings from R2 plant leaves after salt stress

Plasmid	Line #	Electrical conductivity (μmho/mg leaf)	
		Average value[a]	%[b]
pRKJ6 (*Act1-Hva1*)	42	3600±60	63
	44	3830±186	67
	220	4540±138	79
	289	4110±107	71
	Neg[c]	5755±65	100
	NT[d]	5850±35	102
pRKJ21 (*4ABRC-Hva1*)	12	3160±80	55
	13	3360±78	58
	14	3570±40	62
	96	3650±120	63
	Neg[c]	5795±145	100
	NT[d]	5920±300	102

[a] The values are mean from three replicates in each line.
[b] Indicates the percentage of the value of each transgenic plant line and NT over that of Neg control plants, which is set as 100%.
[c] Neg is non-expressing, transgenic line as a control.
[d] NT is untransformed control.

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